European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122
www.biosciencejournals.com
Volume 3; Issue 10; October 2015; Page No. 37-44

Evaluation of antioxidant and antimicrobial activity of *Abies Cilicica* (Ant & Kotschy) subsp. *isaurica coode & Cullen* resin

[1] **Rukiye Yavaşer,** [2] **Huriye Erkuş,** [1] **Çağdaş Sunna,** [1] **Arife Alev Karagözler**

[1] Department of Chemistry, Faculty of Arts and Sciences, Adnan Menderes University, 09010, Aydın, Turkey.
[2] Department of Science Teacher Education, Faculty of Education, Adnan Menderes University, 09010, Aydın, Turkey.

Abstract
In this work resin obtained from cones *of A. cilicica* tree which grows in West Taurus Mountains, in the southern region of Turkey was thoroughly investigated for its antioxidant and antimicrobial activities. Eight different methods (TAC, TPC, reducing power assay, DPPH, CUPRAC, ORAC, TEAC, superoxide radical scavenging) were used to assess antioxidant capacity of *A. cilicica* resin ethanol and water extracts. In general, water extract did not demonstrate high antioxidant activities whereas ethanol extract exhibited high antioxidant activities which were comparable to standard antioxidant compounds such as gallic acid, Trolox, ascorbic acid and α-tocopherol. The results revealed that the utilization of *A. cilicica* resin as a cure for wounds, acne may be due to its antioxidant and antimicrobial properties. It is concluded that resin obtained from *A. cilicica* may be used in ecofriendly and biocompatible pharmaceuticals.

Keywords: *Abies cilicica*, resin, antioxidant activity, antimicrobial activity, radical scavenging

Introduction

Free radicals are generated in human body through various physiological reactions (i.e. respiration) and also after exposure to environmental factors such as air pollution, ionizing radiation, and smoking [1]. These radicals cause oxidative stress which is defined as the imbalance in biological systems when there is an overproduction of free radicals and deficiency of enzymatic or non-enzymatic antioxidants. Oxidative stress may lead to some health problems such as cancer, diabetes, atherosclerosis, Alzheimer, etc. Antioxidants have been accepted as crucial substances for their protective roles on human health against oxidative stress [2].

Since from the ancient times, plants have been evidenced that they have a significant role in preventing and treating diseases. In recent years, apart from their nutritional value, plants and plant products are being attractive due to their antioxidant and antimicrobial properties. Consequently, efforts are being made to explore the antioxidative and/or antimicrobial potential of plants and plant products which are traditionally used for nutrition, alternative medicine and natural therapies. Antioxidative and/or antimicrobial action is attributed mostly to plants' phytochemicals which struggle free radical attacks and reduce oxidative stress. In addition, these phytochemicals may be alternatives for antibiotics since they exert antimicrobial activity.

Resins are naturally occurring products secreted from plants or trees. Generally they are soft, viscous fluids when fresh but hard and glassy after drying. They are oxidation products of essential oils and their chemical compositions are very complex. These hydrocarbon secretions may be used in pharmaceuticals, cosmetic preparations and foods due to their antioxidative and antimicrobial properties. Assimopoulou *et al*. (2005) [3] have investigated the antioxidant activity of natural resins, their essential oils and also bioactive triterpenes (oleanolic acid and ursolic acid) of *Pistacia lentiscus* var. Chia (*P. lentiscus*), *Commiphora myrrh (C. myrrh)*, *Boswellia serrata (B. serrata)*, and *Gum storax (G. storax)* by monitoring the effect on oxidation of lard, corn oil, olive oil and sunflower oil. It was concluded that *P. lentiscus* resin exerted antioxidant activity against oxidation of the oils studied. Essential oils of *C. myrrh* and triterpenes were able to prevent oxidation of sunflower oil while, essential oil of *B. serrata* was active against oxidation of sunflower and olive oil [3].

Gupta and Gupta (2011) [4] have investigated *in vitro* antioxidant and antimicrobial activity of Dragon's blood resin extracts to evaluate their potential to be used as food preservatives. Both of the resin extracts, prepared using ethylene chloride and methanol, served good ability to scavenge nitric oxide, superoxide and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. Extracts were also reported to show significant reducing power. Antioxidant activity was attributed to the high phenolic content of the extracts. Antimicrobial activity was also shown against various food borne pathogens [4].

Abies cilicica (Ant & Kotschy) subsp. *isaurica Coode & Cullen* (*A. cilicica*) is an endemic fir tree that grows in West Taurus Mountains, Turkey. The cones of fir are resinous [5] and the resin is used for treatment of acnes, ulcer, wounds, asthma by local inhabitants since it is supposed to have antiseptic, anti-inflammatory and antioxidant properties. The essential oil composition of young shoots obtained from *A. cilicica* was studied by Bağcı and Babaç (2003) [6] and it was reported that essential oil composition was majorly composed of β-caryophyllene, α-humulene, α-pinene, myrcene and β-pinene [6]. To our knowledge, no through investigation exists in the literature that enlightens the antioxidant properties of *A. cilicica* resin. Therefore, in this work, antioxidant activity of *A. cilicica* resin is evaluated by eight methods for the first time and antimicrobial activity is screened by agar-well diffusion method.

2. Materials and Methods

2.1. Chemicals and Apparatus

Analytical grade chemicals were used in the experiments. Linoleic acid, HCl, NH_4SCN, $FeCl_3$, ammonium acetate (NH_4Ac), dimethyl sulfoxide (DMSO), Mueller Hinton Agar and Caso Agar were obtained from Merck (Darmstadt, Germany). Tetracycline was obtained from Oxoid. $CuCl_2$ and neocuproine were obtained from Aldrich (Milwaukee, WI, ABD). Trolox, $K_3Fe(CN)_6$, ascorbic acid (AA), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), ABTS, $K_2S_2O_8$ were purchased from Fluka (Buchs, Switzerland). Na_2CO_3 and trichloroacetic acid (TCA) was obtained from Carlo Erba (Ronado, Italy). Other chemicals were purchased from Sigma (Steinheim, Germany).

A benchtop shaker (Heidolph, Promax 2020), lyophilisator (Labconco, Freezone 6, Model 77520), rotary evaporator (IKA, RV 05 Basic 1B) were used to prepare extracts. Spectrophotometric measurements were performed on a UV-Vis spectrophotometer (Shimadzu, UV 1601). Distilled water was used for all experiments.

2.2. Resin material

A. cilicica resin was obtained from Alanya-Köprülü village in Antalya, Turkey. The fir tree was identified as *Abies cilicica* (Ant & Kotschy) subsp. *isaurica Coode & Cullen* by botanist Dr. Özkan Eren, in the Biology Department at Adnan Menderes University. *A. cilicica* resin is a viscous, creamy liquid which solidifies when left in the air. Therefore, it is kept in airtight containers at 4 °C until used.

2.3. Preparation of extracts

Two grams of resin was mixed with 20 mL of distilled water in order to obtain water extract. The mixture was shaken at 120 rpm, protected from light, for 2 h at room temperature using a benchtop shaker. After the mixture was filtered through Whatman filter paper No.1, the residue was re-extracted with 20 mL of distilled water for 24h. The filtrates were pooled and frozen. Water was removed by lyophilisation for 36 hours at -50 °C and 0.04 mbar. The solid water extracts were coded with letter (W) and kept at -18 °C in airtight vials until used.

For preparation of ethanol extracts was prepared by using 2.0 grams of resin was shaken with 2x20 mL aliquots of ethanol sequentially for 2 h and 24 h. Resulting solutions were filtered through Whatman No.1 filter paper and the filtrates were pooled. The filtrate was evaporated under vacuum using a rotary evaporator at +40 °C. The residue was transferred to a vial and coded as (E). The ethanol extracts were kept at -18 °C in airtight vials until used.

2.4. Determination of total antioxidant capacity (TAC)

Ferric thiocyanate method was used to determine total antioxidant capacity [7]. One mg/mL solution of extract (W or E) prepared using appropriate extraction solvent was placed in 20 mL screw capped test tube. Then, 1.025 mL of 0.081 M linoleic acid solution in ethanol was added to the test tube. Subsequently, 2.0 mL of phosphate buffer solution (0.04 M, pH 7.4) and 0.975 mL of distilled water were pipetted to the test tube. The resulting emulsion was incubated at +40°C in dark and was used as stock solution. A control without sample was also prepared. One hundred milliliters of stock emulsion, 9.7 mL of ethanol, 100 µL of 20 mM $FeCl_2$ (in 3.5% HCl) and 100 µL of NH_4SCN (30%, w/v) were transferred to 15 mL screw capped test tube and vortexed for 20s. After waiting for 3 minutes, absorbance of the sample was read at 500 nm against a blank which contains no standard antioxidant or extract. Absorbance values that correspond to the time when control reached the maximum oxidation level (72h) were used to calculate inhibition%. Total antioxidant capacity was calculated as percentage inhibition of linoleic acid peroxidation using the equation below:

Inhibition % = $[(A_0-A_s)/ A_0] \times 100$

where A_0 and A_s were the absorbance values of control and sample, at the time that control sample reached its maximum absorbance, respectively.

2.5. Determination of total phenolic content (TPC)

Folin-Ciocalteu method was applied to determine total phenolic content (TPC) of the extracts according to Singleton *et al*. [8]. Stock solutions of extracts were prepared by dissolving 10 mg extract in 10 mL of water or ethanol. Three hundred microliters of stock solution was mixed with 45.7 mL of water in 100 mL flasks. Folin-Ciocalteu reagent (1.0 mL) was directly added. After 3 minutes, 3 mL of Na_2CO_3 (2%, w/v) solution was added. The flasks were stoppered and shaken at 150 rpm in the dark for 2 h at room temperature. Absorbance values at 760 nm were recorded by using distilled water as blank. Gallic acid was used to construct a standard plot. Total phenolic content of extracts was expressed as gallic acid equivalent (µg GAE/mg extract).

2.6. Reducing power assay

The ability of samples to reduce iron(III) to iron(II) was determined using the method reported by Oyaizu [9]. Briefly, 0.5 mL of 1.0 mg/mL extract solution, and 0.5 mL distilled water were mixed. Two and a half mL of 0.2 M PBS (pH 6.6) and 2.5 mL of potassium hexaferricyanide solution (1 %, w/v) were added. After 20 minutes of incubation at +50 °C, 2.5 mL TCA solution (10%, w/v) was added. 2.5 mL of the mixture was transferred to new screw-capped tubes. 2.5 mL distilled water and 0.5 mL $FeCl_3$ solution (0.1%, w/v) was added. The tubes were vortexed for 20 s. Absorbance was read at 700 nm against distilled water as a blank. Ascorbic acid, a compound with strong reducing power, was used as standard. Reducing power of samples was calculated as ascorbic acid %.

2.7. DPPH radical scavenging assay

The DPPH assay was applied according to the method reported by Brand-Williams *et al*. with some modifications [10]. DPPH was dissolved in methanol to obtain 1.0×10^{-3} M radical solution. One mL of $DPPH^{\cdot}$ solution and 3.0 mL of antioxidant solution (0-250 µg/mL in ethanol or water) were pipetted to 10 mL tubes and vortexed for 20 s. Tubes were kept in dark for 30 minutes at room temperature. Absorbance was read at 517 nm. The results were expressed as IC_{50} (µg/mL) values that represent the antioxidant concentration able to scavenge 50 % of DPPH radical.

2.8. Cupric ion reducing antioxidant capacity (CUPRAC) assay

CUPRAC assay was employed according to the method described by Apak *et al*. [11]. Briefly, 0.5 mL of extract solutions (0-100 µg/mL), 0.6 mL solvent, 1.0 mL $CuCl_2$ solution (1×10^{-2} M), 1.0 mL neocuproine solution (7.5×10^{-3} M in ethanol) and 1.0 mL NH_4Ac buffer (1.0 M, pH 7.0) were

added into screw capped test tubes. Tubes were vortexed for 20 s and stored in a dark place at room temperature for 45 min. Absorbance values at 450 nm were recorded. Trolox was used as positive control. Results were expressed as $TEAC_{CUPRAC}$ values by calculating the ratios of slopes of the concentration versus absorbance plots obtained for samples and Trolox.

2.9. Oxygen radical absorbance capacity (ORAC) assay

The capacity of extracts and antioxidants to scavenge peroxyl radicals was determined according to the method reported by Lopez-Alarcon and Lissi [12]. ORAC was assayed using 0.5 mL AAPH (10 mM in 75 mM phosphate buffer pH 7.4) solution, 450 µL extract solution at 0-30 µg/mL concentrations and 50 µL pyrogallol red solution (1.0×10^{-4} M). The decrease of the absorbance was recorded for 15 minutes with 5 minutes intervals. Absorbance at "*t*" minute was proportioned to the absorbance read at "t_0". Time versus A/A_0 values were plotted and degradation curves were obtained. The slopes of these curves (R) were proportioned (R^0/R) to the slope of the curve for the control (R^0) which did not contain antioxidant. Trolox was used as standard and $TEAC_{ORAC}$ values were obtained by calculating the ratios of the slopes of R^0/R plots generated for samples and Trolox.

2.10. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was performed according to Re *et al*. [13]. For TEAC assay, 0.0768 g of ABTS was dissolved in 10 mL of distilled water to obtain 7.0 M solution. This solution was mixed with 10 mL $K_2S_2O_8$ (final concentration 2.45 M) solution and ABTS radicals ($ABTS^{\cdot}$) were generated. The radical solution was kept in dark at room temperature and stirred (100 rpm) with a magnetic stirrer overnight (16 h). Prior to absorbance measurements, $ABTS^{\cdot}$ solution was diluted with distilled water to give an absorbance of 0.70 ± 0.01 at 730 nm. One mL of diluted $ABTS^{\cdot}$ solution and 0.05 mL resin extract solution (0-500 µg/mL) were mixed in a one mL quartz cuvette. The absorbance at the sixth minute was recorded. Trolox, a Vitamin E analogue, was employed as standard. Results were expressed as $TEAC_{ABTS}$.

2.11. Superoxide radical scavenging activity

Superoxide radical scavenging activities of samples were determined by the NADH oxidation according to the method reported by Fu *et al*. [14] and Gülçin *et al*. [15]. Superoxide radicals ($O_2^{\cdot -}$) were generated *in situ* by mixing 1.0 mL of NBT solution (156 µM in 100 mM phosphate buffer, pH 7.4) with 1.0 mL of NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4). Then 100 µL of sample (1.0 mg/mL) and 100 µL of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) were added to start the reaction. Reaction mixture was incubated at 25°C for 5 min. Absorbances at 560 nm were recorded against a blank which doesn't contain PMS.

2.12. Antimicrobial activity

Antimicrobial activities of the extracts of *A.cilicica* resin were determined according to the agar-well diffusion method. All the microorganisms studied were supplied from the Microbiology Division of Biology Department at Adnan Menderes University. Bacteria used were *Staphylococcus aureus (S. aureus)* ATCC25923, *Enterococcus faecalis (E. faecalis)* ATCC51299, *Bacillus cereus (B. cereus)* ATCC11778, *Micrococcus luteus (M. luteus)* ATCC9341, *Escherichia coli (E. coli)* ATCC35218 *and Listeria monocytogenes (L. monocytogenes)* (food isolate). The test was applied by spreading a bacterial inoculum of 1×10^8 cfu/mL on the surface of Mueller-Hinton agar plate. Wells (6 mm diameter) were made using a sterile cork borer. Fifty µL of extract (10 µg/mL in DMSO) dissolved in DMSO was pipetted into the wells. Tetracycline was used as positive control. Plates were incubated at 37 °C for 24 h. The zones of growth inhibition were measured [16].

2.13. Data Processing

Experimental results of this study were mean±SD of three parallel measurements. In each method mean values (n=3) of antioxidant capacities measured in aqueous and ethanolic extracts were compared with the mean values (n=3) of the capacity of individual antioxidant using the t-test in Microsoft Excel. When the calculated t value, without regard to sign, exceeded the critical t value given in tables for a probability level (p=0.05) and for the degrees of freedom estimated assuming unequal variances, it is concluded that there was a significant difference between the two means.

3. Results

Plant kingdom contains a large variety of antioxidant and antimicrobial compounds. Since these compounds differ in molecular structure and act through various reaction mechanisms, such as single electron transfer (SET) and hydrogen atom transfer (HAT), it is crucial to apply more than one method to assess their antioxidant activities. In that perspective, ORAC was chosen as the representative of HAT based method and TAC, TEAC, DPPH, TPC, CUPRAC methods were selected as SET based methods [17]. In addition, antimicrobial activity of extracts towards some microorganisms was investigated. In all experiments well-known natural (i.e. ascorbic acid, gallic acid, α-tocopherol) and synthetic (i.e. BHT, Trolox) antioxidant compounds were used as standard and/or comparison compound.

It is a common procedure to assess the total phenolic content of plant or plant product extracts as a measure of antioxidant quality. Various solvents may be used ranging from polar to nonpolar for extraction of plant material. Since resin mainly contains hydrocarbon derivatives (i.e. terpenes) organic solvents can be appropriate. On the other hand, use of nonpolar organic solvents deteriorates the antioxidant measuring methods which use polar solvents (i.e. water and/or ethanol) as analysis medium. Therefore, use of water or ethanol is a preferred practice to obtain the antioxidant compounds from resinous material [15, 18, 19, 20, 21].

It is difficult to assess each antioxidant individually in a sample extracted from plants. Therefore, it is more reasonable to evaluate the capacity of all potent antioxidants in a complex medium with a convenient method like TAC. Total antioxidant capacity of resin extracts was determined according to the ferric thiocyanate method in which the ability of preventing linoleic acid peroxidation by antioxidant compounds was measured. The high absorbance of $FeSCN^{2+}$ complex at 500 nm means high peroxidation. The results showed that total antioxidant capacities of standards and ethanol extract (E) decreased in order of BHT > α-tocopherol > AA > E > GA (Fig. 1). Ethanol extract's antioxidant capacity calculated as inhibition % was found to be very close to ascorbic acid's

antioxidant capacity (56.8 ± 3.21 versus 59.9 ± 3.68), whereas water extract did not inhibit peroxidation at all. Moreover, ethanol extract exerted higher total antioxidant activity than equivalent amount of gallic acid which is a well-known antioxidant.

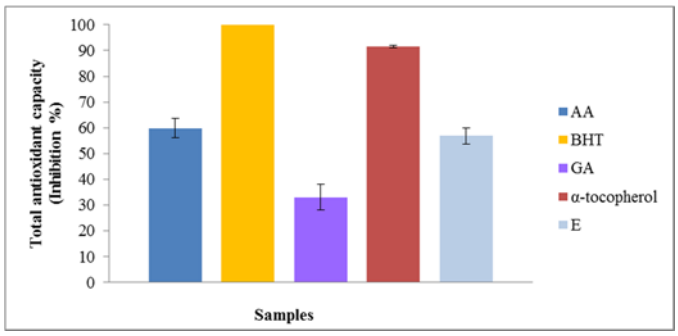


Fig 1: Total antioxidant capacity of ethanol extract of *A. cilicica* and standard antioxidants determined by the ferric thiocyanate method. Data was taken for inhibition of linoleic acid peroxidation at 72 h. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin. Data present means ± SD (n=3)

Phenolic compounds bear one or more aromatic rings substituted with one or more hydroxyl groups. These secondary metabolites are shown to have antioxidant and antimicrobial activities in several researches [18]. The phenolic constituents of *A. cilicica* resin were extracted into water and ethanol and quantified using Folin-Ciocalteu reagent. Gallic acid was used as a representative of phenolic compounds. Total phenolic content results are demonstrated in Table 1. Results showed that ethanol extract possessed 89.2 ± 2.17 µg GAE phenolics while water extract had 21.1 ± 3.34 µg GAE phenolics per one mg extract. It is a common opinion that high phenolic content is related to high antioxidant capacity. Higher antioxidant activity of E than W tested by the methods in this work can be explained by the difference between amounts of phenolic compounds present in the extracts.

Majority of antioxidant compounds are easily oxidizable species and this property makes them good reductants that donate electrons to reduce other compounds. Hence, reducing power of molecules and/or extracts is reported as an indicator of antioxidant activity [22]. Fig. 2 indicates the reducing power of E and W compared to a well-known standard, ascorbic acid. In this assay, antioxidant compounds provide the reduction of Fe^{3+} ions via an electron transfer. The power of standards and samples to reduce Fe^{3+} ions to Fe^{2+} ions decreased in order of AA > BHT > α-tocopherol > E > W. Ethanol extract showed higher reducing power compared to W probably due to higher phenolic content. However, both of the extracts had low reducing power which may be an indication of poor electron donation.

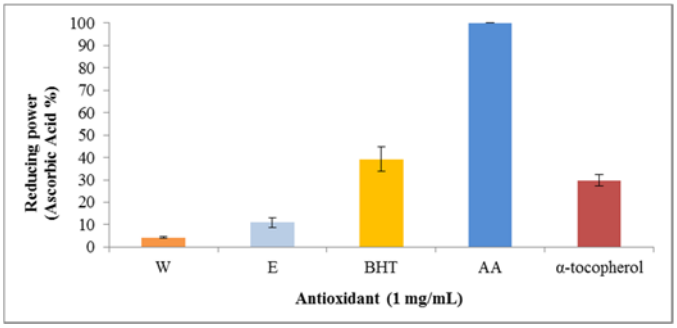


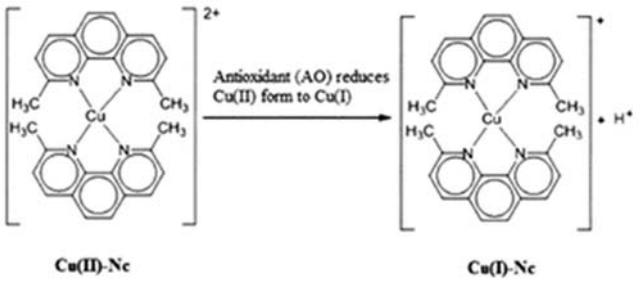
Fig 2: Reducing power of *A. cilicica* extracts and standard antioxidants compared to ascorbic acid. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin, W: water extract of *A. cilicica* resin. Data present means ± SD (n=3)

Table 1: Total antioxidant capacity, total phenolic content and reducing power of *A. cilicica* extracts and standard antioxidants. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin, W: water extract of *A. cilicica* resin. Data present means ± SD (n=3).

Sample	TAC (Inhibition%)	Total phenolic content (µg GAE/mg extract)	Reducing power (AA%)
AA	59.9 ± 3.68	-	100
BHT	100	-	39.3 ± 5.47
GA	33.1 ± 5.04	-	-
Trolox	-	-	-
α-tocopherol	91.6 ± 0.54	-	29.8 ± 2.86
E	56.8 ± 3.21	89.2 ± 2.17	11.02 ± 2.21
W	-	21.1 ± 3.34	4.34 ± 0.317

DPPH is a rapid, economic and simple method for antioxidant activity measurement. It involves measuring the reduction of synthetic radical, DPPH, by antioxidants which can donate a hydrogen atom or an electron. The purple color of DPPH radical solution turns into yellow as the result of reduction and decolorisation is monitored recording absorbance at 517 nm. DPPH radical scavenging activity can be expressed as IC_{50} value corresponding to the concentration of antioxidant compound able to scavenge 50% of DPPH radicals present in the reaction medium. The lower IC_{50} means the higher scavenging activity. In our study, ethanol extract was able to scavenge 70% of DPPH$^{\bullet}$ present in the reaction medium at 250 µg/mL concentration, whereas water extract was able to scavenge 35 % of DPPH radical. However, IC_{50} of ethanol extract (15.9 ± 0.64) corresponded half of the IC_{50} belonging to BHT (8.01 ± 0.36). The results also reveal that BHT is a weak DPPH radical scavenger. Ascorbic acid, gallic acid and α-tocopherol could scavenge DPPH$^{\bullet}$ by 90%.

CUPRAC is a convenient method to detect the antioxidant activity of both hydrophilic and lipophilic compounds. Since the reaction occurs at pH 7.0, the reaction medium is simulated to be close to physiological pH. The assay is based on measuring the absorbance of Cu(I)-neocuproine chelate at 450 nm resulting from the redox reaction of antioxidant compound and CUPRAC reagent as shown below [23].



The capability of E and W extracts to reduce cupric ion (Cu^{2+}) followed a concentration dependent manner (Fig. 3). Results revealed that E had higher $TEAC_{CUPRAC}$ values (1.05 ± 0.143) than GA, Trolox and W, and lower than BHT, AA and α-tocopherol (Table 2). In addition water extract was able to reduce Cu^{2+} ions by a percentage of 60 % of Trolox. The activity of both E and W in this assay can be attributed to the applicability of the assay to lipophilic antioxidants as well as hydrophilic antioxidants.

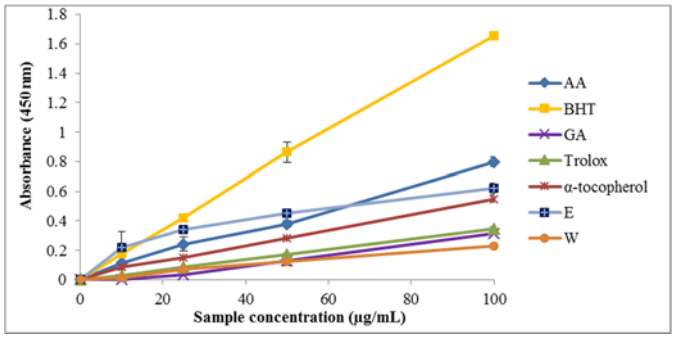


Fig 3: Concentration dependent absorbance values of *A. cilicica* extracts and standard antioxidants at 450 nm in CUPRAC assay. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin, W: water extract of *A. cilicica* resin. Data present means ± SD (n=3)

ORAC method was first introduced by Cao *et al*. (1993) by measuring the fluorescence decay of the protein β-phycoerythrin damaged by peroxyl radicals [24]. In 2005, Lopez Alarcon and Lissi [12] reported a spectroscopic method to determine oxygen radical absorbance capacity. The assay is based on kinetic measurements that involve the consumption of a target molecule (pyrogallol red) by a peroxyl radical generator (AAPH) in the presence of free radical scavengers (antioxidants) at 37 °C, pH 7.0. Since the reaction medium is adjusted to reflect the physiological conditions, results obtained from ORAC might represent *in vivo* effects of antioxidants against peroxyl radicals which are readily generated during normal metabolic reactions in living organisms [25]. According to the $TEAC_{ORAC}$ values obtained in this study, ethanol extract of *A. cilicica* resin exhibited higher peroxyl radical scavenging effect than water extract and the standards. ORAC is a HAT based assay, therefore it can be suggested that ethanol extract of *A. cilicica* resin is rich in compounds capable of donating hydrogen atom. ORAC assay is a method that has been broadly applied both in pure science and the food and supplement industry. Moreover, efforts have been made to build a database by measuring the antioxidant capacity using ORAC assay and total phenolic content [26]. Antioxidant capacity of a compound may be dependent upon reaction media. Therefore, if a method is needed to compare antioxidant capacities of different compounds or plant samples this would be ORAC method since it may be applied to both hydrophilic and lipophilic samples. It can be stated that the most important result of this study is high ORAC property of ethanol extracts of *A. cilicica* resin which puts it in a high antioxidant category [27].

TEAC was employed by measuring the decolorisation of blue-green colored ABTS radical cation chromophore spectrophoto metrically at 730 nm. TEAC is capable of testing the scavenging effect of both hydrophilic and lipophilic compounds towards $ABTS^{\bullet+}$. E and W exhibited low activity in TEAC assay (Fig. 4).

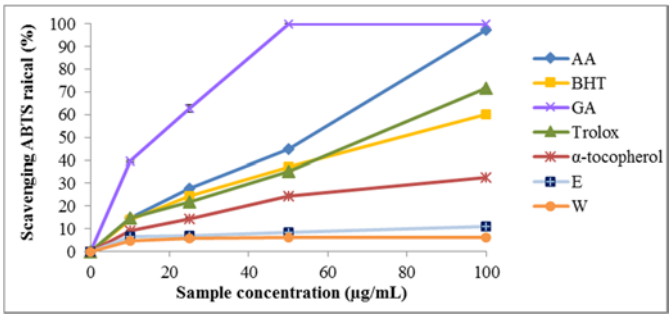


Fig 4: ABTS radical scavenging activity of standards and *A. cilicica* extracts at different concentrations. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin, W: water extract of *A. cilicica* resin. Data present means ± SD (n=3)

Table 2: Total IC_{50}, $TEAC_{CUPRAC}$; $TEAC_{ABTS}$ and $TEAC_{ORAC}$ values of *A. cilicica* extracts and standard antioxidants. AA:ascorbic acid, BHT: butylated hydroxytoluene, GA: gallic acid, E: ethanol extract of *A. cilicica* resin, W: water extract of *A. cilicica* resin. Data present means ± SD (n=3).

Sample	DPPH IC_{50} (µg/mL)	$TEAC_{CUPRAC}$	$TEAC_{ORAC}$	$TEAC_{ABTS}$	Superoxide radical scavenging (Absorbance at 560 nm)
AA	2.65±0.01	2.49±0.567	0.444±0.003	1.335±0.005	0.576 ± 0.084
BHT	8.01±0.36	4.71±0.103	0.388±0.002	0.896±0.002	0.531 ± 0.005
GA	2.77±0.01	1.02±0.009	1.42±0.050	2.974±0.002	0.101 ± 0.011
Trolox	-	1	1	1	0.066 ± 0.003
α-tocopherol	3.5±0.12	1.53±0.059	0.501±0.013	0.515±0.002	0.496 ± 0.016
E	15.9±0.64	1.05±0.143	0.622±0.006	0.181±0.001	0.358 ± 0.033
W	340±1.82	0.65±0.033	0.027±0.022	0.118±0.002	0.356 ± 0.023

Superoxide ($O_2^{\bullet-}$) is a free radical which is readily generated in human body. It is reported to be the precursor leading to generate stronger free radicals than itself such as singlet oxygen and hydroxyl radical [28]. Radicals were generated in a

PMS-NADH system *in vitro*. The decrease in the absorbance of the system indicated higher superoxide radical scavenging ability. At a concentration of 0.1 mg/mL, *A. cilicica* resin extracts were able to exert higher antioxidant activity than AA, BHT and α-tocopherol (Fig. 5). Although BHT and α-tocopherol are known to be compounds, with high antioxidant activity, some plant extracts may have higher activity depending on the method used. Elmastaş *et al*. (2003) reported a similar result for superoxide radical scavenging activity of Marshmallow flower extracts [29].

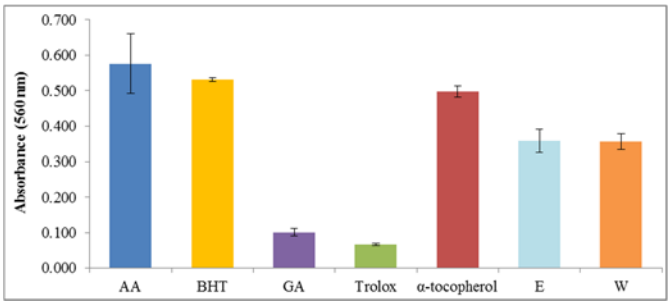


Fig 5: Comparison of superoxide radical scavenging capacities of 100 µg/mL AA, BHT, GA, Trolox, α-tocopherol, E and W. Data present means ± SD (n=3)

Including the comparison between aqueous and ethanolic extracts, a total of 64 comparisons were made. Sixty out of 64 pairs of means were found significantly different. Those that were inferred as not being different were antioxidant capacity of E and AA, E–GA, E-Trolox in the method of CUPRAC, and E-AA in the method of TAC.

Antioxidant capacity values of aqueous and ethanolic extracts may indicate whether there is a contribution to the measured capacity other than phenolic compounds. Table 3 shows the antioxidant capacities of aqueous and ethanolic extracts measured with each method together with phenolic contents of the two extracts. Since the range of values is very large a convenient method for visual comparison is to normalize values by assuming that, in each method, antioxidant capacities measured in ethanolic extracts is always 100.

Table 3: Experimental and normalized antioxidant capacities and phenolic contents of E and W

Method	Experimental Capacities (mean,n=3)		Normalized capacities	
	W	E	W	E
Phenolic content	21.11	89.22	23.66	100
Reducing Power	4.34	11.02	39.38	100
ORAC	0.027	0.622	43.41	100
CUPRAC	0.6476	1.0571	61.26	100
ABTS	0.118	0.181	65.19	100
Superoxide Radical Scavenging	0.355	0.3577	99.25	100
DPPH	340.57	15.85	2148.7	100

Fig 6: depicts the change of the antioxidant capacity of the aqueous extracts measured with individual methods with respect to the capacity of ethanolic extracts (which is assumed to be 100). Visual inspection of the figure renders the conclusion that starting from the method of reducing power each method towards the right of y-axis contribution of compounds other than phenolic ones gets gradually higher, contribution in DPPH being the highest.

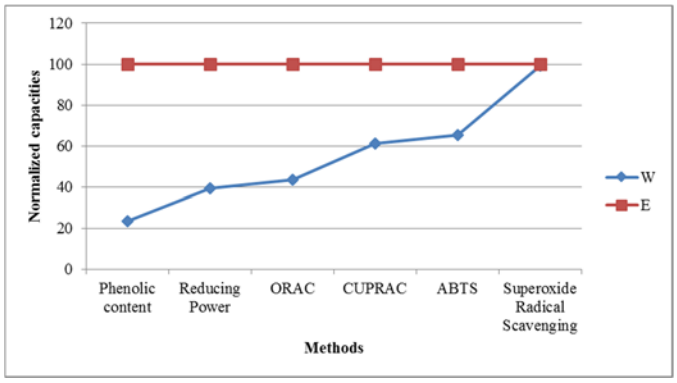


Fig 6: Comparison of relative antioxidant capacities of E and W extracts measured using various methods

In order to seek whether there is a correlation between the antioxidant capacities of measured in aqueous and ethanolic extracts, Spearman correlation coefficient was calculated, excluding the DPPH value, using Minitab. Since the calculated value, excluding the normalized capacity of DPPH, of 0.997 is greater that the critical value of 0.886 (n=6) it is concluded that capacities measured in the two extracts correlate well.

According to the results of antimicrobial activity test, ethanol extracts were found to inhibit the growth of *S. aureus* (causes acnes), *M. luteus* (causes infection on skin), *B. cereus* (causes foodborne disease). These results may explain the traditional uses of *A. cilicica* resin for healing acnes, wounds and ulcers. No antimicrobial activity was detected against *E. faecalis*, *E. coli*, *L. monocytogenes*. Diameter of zones of inhibition was measured (Table 4). Inhibition of bacteria growth by water extracts wasn't detected on the studied microorganisms. Antimicrobial activity of essential oil obtained from *Abies cilicica* (Ant. et Kotschy.) subsp. *cilicica* Carr. which is also a member of *Abies* genus was investigated by Dayısoylu *et al.* (2009) [5]. According to their data reported, the essential oils extracted from cones rosin (4 µL/disc) showed inhibitory effect on growth of all bacteria and yeasts studied including *S. aureus*, *M. luteus* and *B. cereus*. No inhibition was observed in *E. coli* which is consistent with the result in this study.

Table 4: Antimicrobial activity of ethanol extracts (10 µg/mL) of *A. cilicica* resin

Microorganisms	Diameter Inhibition Zone (mm)
S. aureus	11
M. luteus	18
B. cereus	17
E. faecalis	-
E. coli	-
L. monocytogenes	-

4. Conclusion

Studies on resin and/or rosin (hardened form of resin) are mainly concentrated on antimicrobial tests [5] and determination of individual essential oils [20]. Therefore, the studies that investigate the antioxidant properties of resin extracts are scarce and there is not much data to use for comparison. However, studies indicate that resins are rich in terpenes and ethanol extracts of rosin mat contain terpenoids,

steroids, tannins, glycosides, anthraquinones, saponins, flavonoids, alkaloids, carbohydrates, phenols and reducing sugars [19]. In this study *A. cilicica* resin water and ethanol extracts were tested for their antioxidant and antimicrobial activities for the first time. Water extracts did not exhibit strong antioxidant activities as expected since resin constituents (i.e. terpenes) are not soluble in water. The antioxidant measurements were performed using 8 different methods which each measures a different feature of the antioxidant tested. For example, radical scavenging activity was measured using synthetic radicals (i.e. DPPH, CUPRAC, ABTS) which do not exist in living systems but considered to be reliable antioxidant tests and using natural radicals (i.e. superoxide radical) which are produced during natural biochemical processes in living systems. In general, ethanol extract exhibited high antioxidant activity with methods using natural radicals. Also, high total antioxidant capacity test is an accepted measure of being good antioxidant. Additionally, ethanol extract showed antimicrobial activity towards some bacteria. *A. cilicica* resin finds a lot of usage in healing wounds, acnes, and even finds usage as an inhalant or stomach suppressor. It is probable that these practices are results of its antioxidant properties. Consequently, it may be concluded that to benefit from high antioxidant activity, *A. cilicica* resin may be used in ecofriendly and biocompatible pharmaceuticals.

Acknowledgments

The authors thank to Dr. Özkan Eren for identifying *A. cilicica,* Dr. Gamze Başbülbül for providing guidance to perform antimicrobial activity studies and to Coordinatorship of Scientific Research Projects (BAP) of Adnan Menderes University for supplying all the chemicals and standard antioxidants to employ the experiments by project: FEF-10011.

References

1. Gulcin I, Kufrevioglu OI, Oktay M, Büyükokuroglu ME. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.), Journal of Ethnopharmacology. 2004; 90:205-215.
2. Carocho M, Ferreira ICFR. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food and Chemical Toxicology 2013; 51:15-25.
3. Assimopoulou AN, Zlatanos SN, Papageorgiou VP. Antioxidant activity of natural resins and bioactive triterpenes in oil substrates. Food Chemistry 2005; 92:721-727.
4. Gupta D, Gupta RK. Bioprotective properties of Dragon's blood resin: *In vitro* evaluation of antioxidant activity and antimicrobial activity. BMC Complementary and Alternative Medicine 2011; 11:13.
5. Dayısoylu KS, Duman AD, Alma MH, Digrak M. Antimicrobial activity of the essential oils of rosin from cones of *Abies cilicica* subsp. *cilicica*. African Journal of Biotechnology 2009; 8(19):5021-5024.
6. Bağcı E, Babaç MT. A morphometric and chemosystematic study on the Abies Miller (Fir) species in Turkey, Acta Botanica Gallica: Botany Letters 2003; 150(3):355-367.
7. Saha K, Lajis NH, Israf DA, Hamzah AS, Khozirah S, Khamisa S *et al*. Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants, Journal of Ethnopharmacology. 2004; 92:263-267.
8. Singleton VL, Orthofer R, Lamela-Raventos RM. Methods in Enzymology 1999; 299:152-178.
9. Oyaizu M. Studies on products of browning reaction: oxidative activities of products of browning reaction prepared from glucoseamine. Japanese Journal of Nutrition. 1986; 44:307-315.
10. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensmittel-Wissenschaft und Technologie 1995; 26:25-30.
11. Apak R, Güçlü K, Özyürek M, Karademir SE, Altun M. Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: the CUPRAC method. Free Radical Research 2005; 39(9):949-961.
12. Lopez-Alarcon C, Lissi E. Interaction of pyrogallol red with peroxyl radicals. A basis for a simple methodology for the evaluation of antioxidant capabilities. Free Radical Research 2005; 39(7):729-736.
13. Re R, Pellegrini N, Protrggente A, Pannala A, Yang M. Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26:1231-1237.
14. Fu W, Chen J, Cai Y, Lei Y, Chen L, Pei L *et al*. Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch. et Sav.) Ching, Journal of Ethnopharmacology. 2010; 130:521-528.
15. Gülçin İ, Büyükokuroğlu ME, Oktay M, Küfrevioğlu Öİ. Antioxidant and analgesic activities of turpentine of *Pinus nigra* Arn. subsp. *pallsiana* (Lamb.) Holmboe, Journal of Ethnopharmacology. 2003; 86:51-58.
16. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966; 45(4):493-496.
17. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, Journal of Agricultural and Food Chemistry. 2005; 53:4290-4302.
18. Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules 2010; 15:7313-7352.
19. Yehia AA, Alzowahi FAM, Kadam TA, Shaikh RU. *In vitro* evaluation of antimicrobial and antioxidany activity of Dragon's blood tree (*Dracaena cinnabari* Balf.f.) of Socotra island (Yemen). Journal of Coastal Life Medicine. 2013; 1(2):123-129.
20. Ulukanlı Z, Karabörklü S, Bozok F, Ateş B, Erdoğan S, Cenet M *et al*. Chemical composition, antimicrobial, insecticidal, phytotoxic and antioxidant activities of *Pinus pinea* resin essential oils, Chinese Journal of Natural Medicines. 2014; 12(12):901-910.
21. Modak B, Salina M, Rodilla J, Torres R. Study of the chemical composition of the resinous exudates isolated from *Heliotropium Sclerocarpum* and evaluation of the antioxidant properties of the phenolic compounds and the resin. Molecules 2009; 14:4625-4633.
22. Karagözler AA, Erdağ B, Emek YÇ, Uygun DA. Antioxidant activity and proline content of leaf extracts

from *Dorystoechas hastata*. Food Chemistry 2008; 111:400-407.
23. Apak R, Güçlü K, Özyürek M, Karademir SE. A novel total antioxidant capacity index for dietary polyphenols, vitamin C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. Journal of Agricultural and Food Chemistry 2004; 52:7970-7981.
24. Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biology and Medicine 1993; 14:303-311.
25. Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, Hampsch-Woodill M *et al*. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$) of plasma and other biological and foodsamples, Journal of Agricultural and Food Chemistry. 2003; 51:3273-3279.
26. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays, Journal of Agricultural and Food Chemistry. 2005; 53:1841-1856.
27. Sanchez-Moreno C. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International 2002; 8(3):121-137.
28. Gülçin İ. Antioxidant activity of food constituents: an overview. Archives of Toxicology 2012; 86:345-391.
29. Elmastas M, Ozturk L, Gokce I, Erenler R, Aboul-Enein HY. Determination of antioxidant activity of Marshmallow flower (*Althaea officinalis* L.). Analytical Letters 2004; 37(9):1859-1869.